Regulation of Synthesis of Non-Globin Proteins in Cell-free Extracts of Rabbit Reticulocytes*

(Received for publication, November 9, 1972)

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SUMMARY

Whole rabbit reticulocytes synthesize, in addition to globin, six predominant protein species. Lysates, free of whole cells or membranes, synthesize precisely the same proteins in nearly the same relative amounts. In cell-free extracts initiation of synthesis of all non-globin and globin species is inhibited by deprivation of hemin or by addition of 10⁻² μg per ml of double-stranded RNA. Similarly, translation of an exogenous viral mRNA, prepared from reovirus, also requires hemin and also is inhibited by double-stranded RNA. Experiments using inhibitors of polypeptide chain elongation indicate that the amounts of mRNAs coding for the non-globin proteins do limit the amount of synthesis of these proteins. Furthermore, individual molecules of mRNA which code for certain non-globin proteins can initiate protein synthesis as much as four times as often as do those which code for other reticulocyte proteins.

Over 90% of protein synthesized by rabbit reticulocytes is hemoglobin. Since these cells do not contain a nucleus and make no RNA, any regulation of protein synthesis must occur at the level of translation. Cell-free extracts from these cells synthesize globin, and, as we shall show, non-globin proteins for long periods of time at rates comparable to those of the intact cell. This is an ideal system for biochemical studies on control processes at the translational level.

Recent work has identified several factors which regulate globin synthesis in extracts of these cells as well as in the intact cell. First, initiation of synthesis of both chains of globin in intact reticulocytes requires the presence of hemin (1, 2); under conditions of iron or of hemin deficiency the polyribosomes disaggregate, indicating that the inhibition is at the level of initiation of globin synthesis (3–5). Globin synthesis in reticulocyte lysates also requires hemin (6, 7); apparently, an inhibitor of chain initiation is formed by the ribosome-free S 100 in the absence of hemin but not in its presence (8, 9). Neither the nature of the inhibitor nor its site of action is known.

Second, the cells exhibit a complex regulation so as to synthes...
Each of the non-globin proteins is synthesized in different relative amounts, but all are made at a rate much less than that of globin. Since, over a long period of time the amount of protein produced is proportional to the product of the amount of appropriate mRNA and the frequency at which ribosomes initiate translation of the mRNA, we attempted to determine which of these factors limits the rate of synthesis of each of the non-globin proteins. Our experiments, utilizing inhibitors of polypeptide chain elongation, indicate that the amount of mRNA for each of the non-globin proteins are indeed present in much lower amounts than are those for globin. However, the mRNAs for the different non-globin proteins differ by up to factors of four in the relative ability to initiate protein biosynthesis.

MATERIALS AND METHODS

Pacltaxmycin was a generous gift from C.I.B.A. Double-stranded RNA, isolated from *Penicillium chrysogenum*, was a generous gift from Dr. Hugh Robertson of the Rockefeller University, New York, N. Y. Reovirus mRNA was donated by Mr. Max McDowell and Dr. W. Joklik of Duke University, and was prepared as detailed previously (23, 24). Hemin was purchased from Schwarz-Mann; [55]methionine (30,000 mCi per mmole) from Amersham-Searle; and [3H]leucine, used at specific activity of 130 mCi per mmole, from New England Nuclear Corp. Sources of all other chemicals were described previously (16, 25, 26).

**Cell-free Protein Synthesis**—Preparation of reticulocytes and their lysates and conditions for cell-free protein synthesis have been described previously (16, 25, 26). Each extract was tested in detail for the optimum concentration of magnesium acetate (1.5 mM to 2 mM for all lysates used here) and for hemin (20 µg per ml for all lysates). Reactions generally contained 19 non-radioactive amino acids (10⁻⁴ M each) and one radioactive amino acid, [55]methionine (80 µCi per ml) or [3H]leucine (14 µCi per ml). Incubation was at 30°C; irrespective of the radioactive label, incorporation was linear for at least 40 min. All counting of radioactive precipitates was done in a low background gas-flow counter with a counting efficiency of 22% for both 3H and 55S. For analysis on polyacrylamide gels, ribonuclease (100 µg per ml) was added and samples were incubated at 37°C for 5 min.

**Labeling of Whole Reticulocytes**—Reticulocytes were collected and incubated in a medium (6) containing also [55]methionine (50 µCi per ml) and 19 non-radioactive amino acids, 2.5 × 10⁻⁴ M each. Incubation was at 30°C for 40 min, during which time incorporation was linear. Reactions were stopped by chilling in ice; the cells were recovered by centrifugation, washed three times in 0.9% sodium chloride solution, and then resuspended directly in the sample buffer used for gel electrophoresis. The cells were completely lysed, and there was no visible precipitate after centrifugation at 15,000 × g for 10 min.

**Acrylamide Gel Electrophoresis**—Acrylamide gels (7.5%) containing 0.1% sodium dodecyl sulfate and 6 x urea were used as described (27). Samples, either about 0.01 ml of packed cells or 0.05 ml of cell-free reaction, were added to 0.2 ml of sample buffer which contained, per liter: Tris, 0.6 g; glycine, 2.8 g; urea, 360 g; sodium dodecyl sulfate, 10 g; 2-mercaptoethanol, 20 ml. After heating to 100°C for 2 min, the samples were subjected to electrophoresis for 20 hours at 35 volts (about 4.5 ma per gel). Gels were stained with Coomassie blue and destained as described by Fairbanks et al. (28) except that no Coomassie blue was added to the destaining solutions. The gels were then cut longitudinally into four slices; the two center slices were dried and subjected to radioautography (Kodak RB-54 film) for periods of time indicated in the individual experiments. Radioautograms were scanned with a Joyce-Loebel microdensitometer with a wedge such that full-scale pen deflection was about 1.2 O.D. units.

**RESULTS**

**Kinetics of Cell-free Protein Synthesis**—When supplemented with 20 µg of hemin per ml, our reticulocyte lysates synthesize protein at 30°C at a linear rate for at least 40 min (Fig. 1). The time required for synthesis of a complete globin molecule is within a factor of two that of an intact reticulocyte (10). If hemin is omitted, or if double-stranded RNA (10⁻⁴ µg per ml) is added, protein synthesis is normal for about 8 min and then ceases (Fig. 1), confirming results of others (6, 7, 17, 18). It should be pointed out that for 8 min there is considerable initiation of protein synthesis in the presence of double-stranded RNA or in the absence of hemin; Fig. 1 also shows that pacltaxmycin, at a concentration which completely blocks polypeptide chain initiation but does not affect polypeptide chain elongation (26), allows protein synthesis for only 1½ min, the time required to complete the globin chains which were nascent at the time the extracts were prepared (10, 26).

**Synthesis of Non-globin Proteins—**Polyacrylamide gel electrophoresis in the presence of urea and sodium dodecyl sulfate was used to resolve the non-globin proteins produced by these
lysates. The gels were sliced and submitted to radioautography; the films were scanned with a microdensitometer. Fig. 3b shows that at least six well defined non-globin proteins (Bands A to F) were synthesized by a reticulocyte lysate supplemented with hemin. In some experiments (Fig. 3g) an additional band is present between E and F. Fig. 2a shows that intact reticulocytes synthesize precisely the same non-globin polypeptides (as well as A- and B-globin, of course). The approximate molecular weights of these proteins, calculated from their mobilities in the gel relative to those of standard proteins, are given in Table I. We do not know the identity of most of these non-globin protein species, although recent work suggests that two of them (B and E) are membrane proteins. Although polypeptide A has the same molecular weight as hemoglobin, it is not an aggregate of globin polypeptides; the gels contain urea, sodium dodecyl sulfate, and mercaptoethanol, which will dissociate such complexes. Fur-

\[1 \text{H. F. Lodish, manuscript in preparation.} \]
Molecular weights of reticulocyte proteins synthesized in cell extracts

Molecular weights of the radioactive proteins (Fig. 2) were calculated from their mobility in sodium dodecyl sulfate-urea polyacrylamide gels by extrapolation from the mobilities of marker proteins run in parallel gels. The marker proteins used (Fig. 2) were: (1) lysozyme, mol wt = 14,400; (2) trypsinogen, mol wt = 23,800; (3) reovirus σ1 protein, mol wt = 34,000 (Reference 45); (4) reovirus σ2 protein, mol wt = 36,000; (5) α-amylase, mol wt = 48,000; (6) bovine serum albumin, mol wt = 64,000; (7) reovirus μ protein, mol wt = 80,000, (Reference 45). A plot of the log of molecular weight versus the mobility of the marker proteins yielded a straight line.

Gel band Molecular weight
A 64,000
B 56,000
C 45,000
D 39,000
E 33,000
F 20,000

At some time up to 40 min. Comparing f and d, it is apparent that, in the absence of hemin, there was no initiation of synthesis of any of the non-globin proteins A to F or of globin after 10 min of incubation. By contrast, in the presence of hemin there is appreciable initiation (and completion) of all protein species throughout the reaction.

We conclude that the absence of hemin has no effect on initiation or synthesis of any protein—either globin or non-globin—for the first 10 min, but after this time the absence of hemin results in cessation of initiation of synthesis of all protein species.

Requirement of Hemin for Translation of Exogenous Viral RNA—Previous work showed that when reovirus messenger RNA is added to a reticulocyte lysate, there is synthesis of large amounts of all eight known reovirus-specific proteins (24). Four of these proteins (σ1 to σ4) have molecular weights of about 30,000, two (μ and μ1) of 50,000, and two (λ1 and λ2) of about 130,000. In the experiment depicted in Fig. 4b, reo mRNA was added to a hemin supplemented lysate at 10 min; after a further 30-min incubation, there is synthesis of both σ and μ proteins, although the individual proteins within the two classes are not resolved due to the short electrophoresis time. (This preparation of reo mRNA apparently is deficient in ability to synthesize the largest λ reo proteins.) Fig. 4c, by contrast, shows that a lysate which has been incubated for 10 min in the absence of hemin will not synthesize any reo virus proteins. Other experiments not shown indicated that when reo mRNA is added at the beginning of incubation to a lysate unsupplemented with hemin, there is, by 40 min, synthesis of some reo proteins, but less than in the presence of hemin. We conclude that a lysate incubated for 10 min in the absence of hemin is unable to initiate synthesis of either virus- or cell-specific proteins.

Effect of Double-stranded RNA—Addition of 10^{-3} μg per ml of double-stranded RNA results in inhibition of synthesis of globin and of all major non-globin proteins (Fig. 5). As is the case with heme deprivation, synthesis of the very largest proteins (X and Y) is largely unaffected, presumably because most of these proteins whose synthesis was completed during the 40-min incubation were nascent when the extract was prepared. Also similar to the effect of heme deprivation (compare Fig. 5, b and c), synthesis of the larger non-globin proteins A, B, and C is less inhibited by double-stranded RNA than is synthesis of globin. Again, this is expected since the mRNAs for A, B, and C, being presumably larger than that for globin, will contain more ribosomes. Hence, when the block in polypeptide chain initiation is manifest at 8 to 10 min (Fig. 1) they will produce more completed polypeptide chains than will globin mRNA. In other words, this experiment indicates that synthesis of all proteins which were made in appreciable amounts in the presence of double-stranded RNA was initiated during the first 8 min of incubation when double-stranded RNA, like hemin deprivation, has no effect on polypeptide chain initiation.

Fig. 4d shows that translation of exogenous reo mRNA added after 10 min incubation, is totally abolished when 10^{-2} μg per ml of double-stranded RNA is added at the beginning of the reaction.

These experiments utilized double-stranded RNA isolated from P. chrysogenum. Similar results were obtained with poly(I)-poly(C) (data not shown).
which the mRNA attaches to ribosomes and initiates protein biosynthesis. This relationship is true even if the rates of elongation of the different proteins are different. We previously showed however, that the rates of elongation of the α- and β-globin chains are identical (10), and it is an implicit and critical assumption in the following experiment that the rates of elongation of all proteins in the reticulocyte—measured in amino acids polymerized per min—are the same.

The present experiments utilize three antibiotics which, at partially limiting concentrations, reduce the rate of propagation of nascent protein chains but which do not directly inhibit initiation of new chains. This results in a large increase in polysome size as each mRNA becomes saturated with ribosomes. Under these conditions protein biosynthesis is not limited by the rate of chain initiation and the relative yields of the different proteins should more nearly approximate the relative amounts of the mRNAs. The assumptions and limitations of this technique are detailed in our earlier paper on the relative amounts of α- and β-globin mRNA (11). Fig. 6 shows the effect on synthesis of globin and non-globin proteins of the two antibiotics emetine (9 X 10^-7 M) and anisomycin (2 X 10^-7 M and 4 X 10^-7 M) at concentrations which inhibit over-all protein synthesis by 60%, 47%, and 39%, respectively. It is apparent from the figure that both drugs inhibit synthesis of certain of the proteins much more than others. For instance, production of protein F is inhibited hardly at all whereas globin synthesis is reduced 3-fold (compare Fig. 6, d and a); likewise, synthesis of E and D is
Table II

<table>
<thead>
<tr>
<th>Effect of antibiotics on synthesis of reticulocyte proteins</th>
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<tr>
<td>Amount of protein produced</td>
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<tr>
<td>A B C D E F Globin</td>
</tr>
<tr>
<td>arbitrary units</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
</tr>
<tr>
<td>1 None</td>
</tr>
<tr>
<td>2 Emetine (9 x 10^{-7} M)</td>
</tr>
<tr>
<td>3 Emetine (1.8 x 10^{-6} M)</td>
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<tr>
<td>4 Anisomycin (2 x 10^{-7} M)</td>
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<tr>
<td>5 Anisomycin (4 x 10^{-7} M)</td>
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<tr>
<td>6 Sparasomycin (3 x 10^{-7} M)</td>
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Inhibited much less than that of B which, in turn, is inhibited less than that of A. To quantify these effects, the areas from the peaks of the microdensitometer tracings were weighed. Table II shows data from a more complete experiment than that of Fig. 6. While there is some error in these numbers, due both to the difficulty of establishing accurate base-line for the peaks and in incomplete separation of some peaks, the same striking differences in inhibition of synthesis of the different polypeptides were found. Synthesis of protein F was least inhibited by all drugs tested, while production of proteins A and C was most sensitive. The inhibition is clearly not a factor only of the molecular weight of the protein. The experiment in Table II utilized [methyl-3H]leucine; most others in this paper utilized [35S]methionine. We emphasized that the amount of protein measured in this experiment depends on its content of leucine; also the ratio of some non-globin proteins made to that of globin is different when [14C]leucine or [35S]methionine are used (compare with Fig. 6).

Two conclusions can be drawn from these data. First, the absolute amount of protein produced in the presence of inhibiting amounts of antibiotics should be proportional only to the amount of the appropriate mRNA (11). There is more mRNA for protein A than for any other non-globin protein; the ratio of the amount, by weight of mRNA for protein A relative to that for globin, is, from an average of Reactions 2 to 7, 2.5 ± 0.5% (Table II). This means that the amounts of mRNA for the non-globin proteins do limit the synthesis of these proteins.

Second, individual molecules of mRNA coding for different non-globin proteins differ in their ability to initiate protein synthesis. For example, the amount of mRNA coding for proteins C and D, measured by the absolute amount of protein produced in the presence of inhibiting concentrations of any of the drugs tested, is about the same. However, in the absence of any antibiotic about 2.5 times as much C protein is made, a result implying that normally ribosomes will initiate protein synthesis on C mRNA about 2.5 times as often as on D mRNA. Similarly, one can calculate that mRNAs for proteins A and C bind to ribosomes and initiate protein synthesis twice as often as does each globin mRNA.

Discussion

Identity of Non-globin Proteins—Reticulocyte lysates, supplemented with hemin, synthesize protein at a linear rate for at least 50 min (Fig. 1) and synthesize precisely the same proteins—globins and non-globins—in about the same relative amounts as do intact reticulocytes (Fig. 2). This is additional evidence that our lysates reproduce faithfully the synthetic pattern of the intact cell. That our lysates, which contain no membranes or membrane-attached ribosomes, synthesize all proteins made by the intact cell is consistent with the claim of Bulova et al. that certain proteins are made predominantly by ribosomes attached to cell membranes (21).

Our polyacrylamide gels utilize strongly denaturing solutes (6 M urea, 0.1% sodium dodecyl sulfate), and we have been unable to determine the nature of these non-globin proteins. In unpublished studies we have shown that each of these proteins corresponds to a protein band visualized with Coomassie blue stain; however, there are many protein bands of equal staining intensity which apparently are not synthesized by reticulocytes or their lysates.

Effect of Hemin—For the first 8 min of incubation reticulocyte lysates will initiate synthesis of all globin and non-globin proteins, regardless of whether hemin is added. After this point, however, deprivation of hemin results in cessation of initiation of synthesis of all reticulocyte proteins. Hence hemin is required for continued initiation of synthesis of both globin and of all non-globin proteins; the observation that proteins whose synthesis is initiated during the first 8 min of reaction—before the hemin requirement is manifest—can be completed normally thereafter is consistent with the notion that hemin is required only for the initiation step of protein synthesis (6, 7, 9).

Recently, it has been shown that ribosome-free supernatants of reticulocytes contain a "pre-inhibitor protein" which is converted into an inhibitor of initiation of protein synthesis by incubation at 37° (8, 29, 30). Also, several groups showed that the effects of this inhibitor can be overcome by a protein component, presumably an initiation factor present in the 0.5 M KCl wash of reticulocyte ribosomes (31, 32). Hunt et al. in a recent paper (33) have presented evidence questioning the physiological role.
of this inhibitor in the regulation of globin synthesis. They believe that a factor required for initiation of protein synthesis is present in limiting amounts and is inactivated somehow during protein synthesis; according to their work hemin would be required for regeneration of this factor. Hence the exact mechanism of action of hemin is still unsettled.

Nonetheless, the present results indicate that hemin is acting either directly or indirectly at a protein which is required for synthesis of all reticulocyte proteins. The target could be, for instance, an initiation factor required for binding of the initiator met-tRNA \(_f\) (25, 34, 35) or a factor required for binding of all mRNAs. The fact that hemin is apparently not required for protein synthesis by other types of cells implies that the erythroidic cells contain a unique regulatory protein which responds to levels of iron (or hemin) in the environment so as to regulate synthesis of all cellular proteins. That synthesis of non-globin proteins be subjected to the same control process as is globin is clearly advantageous for the cell.

**Role of Double-stranded RNA**—Many types of double-stranded RNAs, both synthetic homopolyribonucleotides and RNAs isolated from cells and viruses, are potent inhibitors of polypeptide chain initiation (17-20). The minimum inhibitory concentration of most antibiotics which inhibit polypeptide chain initiation, such as pactamycin, is approximately 1 molecule per ribosome (calculated from Reference 26). By contrast, much less than 1 molecule of double-stranded RNA per ribosome is sufficient to totally abolish, after an initial lag period, polypeptide chain initiation (18). The implication that double-stranded RNA interacts not with the ribosome but rather with some other element, possibly an initiation factor, in the cell supernatant is supported by recent work of Hunter et al. (18). It is of interest that both in the presence of double-stranded RNA or in the absence of hemin protein synthesis is normal for about 8 min and then ceases (Fig. 1). In this paper we showed that, after a lag of about 8 min, double-stranded RNA inhibits completely initiation of synthesis of all reticulocyte globin and non-globin proteins. This implies that the component which is inhibited by this RNA is required for initiation of synthesis of reticulocyte proteins (Fig. 5). Similarly, translation of an exogenous mRNA, reo mRNA, is also blocked (Fig. 4). Hence hemin deprivation and addition of double-stranded RNA have precisely the same effects on translation of all reticulocyte proteins. However, it is not clear whether the same mechanism is involved in both cases.

Ehrenfeld and Hunt proposed that production of double-stranded RNA after infection by small RNA viruses, such as poliovirus, is the mechanism by which these viruses shut off synthesis of cellular proteins. For this to be the case translation of cellular mRNAs, but not viral RNA, must be sensitive to double-stranded RNA. Robertson and Mathews showed that, in a cell-free system from mouse ascites cells, translation of globin mRNA and EMC viral RNA were equally sensitive to inhibition by double-stranded RNA, although inhibition required concentrations of RNA higher than required to inhibit reticulocyte synthesis (20). (They indicated that this may be due to a potent double-stranded RNA nuclease in the ascites extract.) The implication from these results that translation of viral RNA is not more resistant to double-stranded RNA than cellular mRNA is supported by the present observation that in reticulocyte lysates reo mRNA and globin mRNA translation are equally sensitive to inhibition by double-stranded RNA. It is not clear, however, that globin (or other reticulocyte mRNAs) is a typical example of cellular RNA, so it is perhaps premature to conclude that double-stranded RNA is not specific for translation of cellular mRNAs.

**Messenger RNAs for Non-globin Proteins**—Reticulocyte polioviruses contain, in addition to ribosomal RNA, 5 S RNA, and tRNA, large amounts of the RNA species (9 S RNA) which has been shown conclusively to be the messenger for \(\alpha\) - and \(\beta\)-globin polypeptides (36-40). Reticulocyte polioviruses also contain significant amounts of a 17 S RNA and also other RNA species; it is not clear whether these are precursors of the 9 S globin mRNA, are messengers for non-globin proteins, or have some other function (41, 42).

In the present study we have taken, as measure of the relative amounts of functional mRNA for the different globin and non-globin proteins, the amounts of protein synthesized in the presence of inhibitors of polypeptide chain elongation. One conclusion (Table II, Fig. 6) is that the amounts of mRNA for the non-globin proteins do limit their synthesis; there is more mRNA by weight, of protein \(A\) than of any other non-globin protein but the ratio of the amount, by weight, mRNA for \(A\) to that of globin is only about 2%. In these experiments we assumed that the rate of elongation of all of the reticulocyte proteins is the same. Other limitations on this technique of measuring relative amounts of mRNA have been detailed in a previous publication (11). Similar techniques have been used to measure the amount of functional mRNA in a cell during mitosis (43) and the relative amounts of mRNAs for different adenovirus proteins (44).

Another conclusion is that individual molecules of reticulocyte messenger RNA, coding for different proteins, differ in their relative ability to bind to ribosomes and initiate protein biosynthesis. We showed previously that a molecule of \(\beta\)-globin mRNA will, over a period of time, direct initiation of synthesis of about 50% more globin chains than will an \(\alpha\) mRNA (10, 11). Similarly, we show here that mRNAs for proteins \(A\) and \(C\) will initiate protein synthesis twice as often as will a globin mRNA and about four times as often as will a mRNA for protein \(F\).

A major result of this work is that there exist two types of regulation of translation of reticulocyte mRNAs. First, different mRNAs have intrinsically different rates of initiation of protein synthesis. Possibly this is due to different secondary or tertiary structure about the (presumptive) AUG initiation codon (25, 34, 35); possibly this is caused by different primary nucleotide sequences near the initiation codon. Whether or not the relative rates of translation of different mRNAs change during erythrocyte maturation or as a result of other environmental changes is not known at this time; nor is it known whether specific proteins are required for translation of the different reticulocyte mRNAs. By contrast, two treatments—deprivation of hemin or addition of minute amounts of double-stranded RNA—inhibit synthesis of all reticulocyte proteins to the same extent. Hence at least one step common to initiation of synthesis of all reticulocyte proteins is subject to regulation in the cell.

**Acknowledgments**—We thank Dr. Hugh Robertson for a gift of \(P.\) chrysogenum double-stranded RNA, Drs. Max McDowell and W. Joklik for a gift of reo mRNA, Dr. Jon King for instruction on the use of the microdensitometer, and Drs. Hugh Robertson, Tim Hunt, and Mike Mathews for communicating their results before publication.

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